RNAi Pesticides: Toward Hornworm Control via Bacterial dsRNA

Presented by Elizabeth Catherine Robinson

In partial fulfillment of the requirements for graduation with the Dean's Scholars Honors Degree in Biology

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Abstract

Conventional chemical insecticides frequently have negative off-target effects on other organisms. Both important pollinators and human consumers have been harmed due to insecticide consumption. Some researchers have proposed using RNA interference (RNAi) to target essential genes that are specific to agricultural pests: if a pest insect ingests double-stranded RNA (dsRNA) with a sequence identical to an important gene, RNAi can reduce expression of that gene, thereby killing the pest. This could mitigate harmful effects on other insects or humans, because genes specific to one pest species can be targeted. However, dsRNA is unstable outside of living cells, and would quickly deteriorate if simply sprayed over a crop field. Instead, it may be possible to modify bacteria that naturally live on crop plant leaves to produce the dsRNA continuously. Here, I establish the necessary background for using this insecticide alternative against the tobacco hornworm, *Manduca sexta*. I confirm that a non-pathogenic strain of the common crop symbiont, *Pseudomonas syringae* (PSY), colonizes tomato leaves to high titers for at least 72 hours, suggesting that this species could remain effective at producing dsRNA for long periods of time. I construct two plasmids that express dsRNA for portions of the *M. sexta* vascular *ATPase* gene, which is essential to *M. sexta* survival, and transform them into two strains of PSY. I integrate a gene encoding expression of a green fluorescent protein (GFP) into both PSY genomes so that colonization can be tracked more easily. Finally, I attempt to replicate a study that increased tobacco hornworm mortality upon ingestion of *vATPase* dsRNA—however, this replication was unsuccessful, likely due to large numbers of *M. sexta* caterpillars dying of an infectious disease. Overall, these results support the concept that using symbiotic leaf bacteria to continuously produce and stabilize insecticidal dsRNA may be feasible. The tools I have developed suggest that trials on live pests and live plants may be just around the corner. In the future, the stability, sustainability, and cost effectiveness of dsRNA insecticides could allow us to protect crop plants without sacrificing human or environmental health.

Background

Large-scale use of insecticides optimizes agricultural output by preventing devastation from crop pests. However, conventional chemical insecticides frequently have off-target effects on other, non-pest organisms, such as bees or other insects that may be beneficial to agriculture or natural ecosystems (Mansour et al., 2018). Additionally, growing insect resistance to chemical insecticides threatens to outpace the creation of new ones (Gould et al., 2018). Most importantly, insecticide residues on produce have been implicated in numerous human health issues, acting as carcinogens, endocrine disruptors, or neurotoxins (Kalliora et al., 2018). Generalized insecticides with off-target effects have proven to be a double-edged sword, harming humans even as they help secure our food supply.

To eliminate off-target effects in other species, some researchers have proposed manipulating RNA interference (RNAi) to target genes in specific insects. Eukaryotes have evolved RNAi as an antiviral defense system capable of recognizing double-stranded RNA (dsRNA) and cleaving any messenger RNA (mRNA) with a matching sequence (Scott et al.,

2013). By synthesizing dsRNA to match the sequences of essential genes in pest insects, and subsequently having the insects ingest them, their cells can be tricked into cleaving their own mRNAs for genes essential to survival. One major hurdle preventing the use of dsRNA in crop fields is the chemical instability of RNA: insects are unlikely to ingest dsRNA that has been sprayed onto a crop field before it degrades (Whyard et al., 2009).

Some researchers have looked towards engineering laboratory strains of *E. coli* to produce the dsRNA, as bacteria could provide a long-lasting source of dsRNA (Bento et al., 2020; Vatanparast & Kim, 2017). While *E. coli* is well-studied and easy to rear in a laboratory setting, it is not a natural plant symbiont and therefore is unlikely to sustain high-density colonies on crop leaves for an extended period of time. A longer-lasting solution would be to engineer strains of bacteria adapted to live on the leaf surfaces of crop plants. My research aimed to engineer bacteria to produce dsRNA for RNAi, but with non-pathogenic plant symbionts that already colonize crop leaves instead of *E. coli*. Non-invasive, non-pathogenic species would be unlikely to hurt crops or consumers—as humans already eat produce with leaves bearing these species—and may colonize the plants more efficiently and sustainably.

I tested a proof-of-concept system using tomato plants because of their agricultural importance and ability to grow in a laboratory. The bacteria species *Pseudomonas syringae* (PSY) 508 and 642 colonize a wide variety of plants without any apparent pathogenic effects (Clarke et al., 2010), making them ideal symbionts. The tobacco hornworm (*Manduca sexta*) decimates tomato crops (as well as other plants in the *Solanaceae* family, such as tobacco and eggplant), making it an ideal target for this study. Previous research shows that ingested dsRNA can kill these caterpillars by targeting *vATPase* subunit E, which encodes part of a vascular proton pump necessary for hornworm survival (Whyard et al., 2009). *M. sexta vATPaseE* has a low amount of homology in other related insects, the highest matches being with the cotton bollworm and the cabbage looper, which are also agricultural pests. This makes it a perfect candidate for testing species-specific targeting, as low homology indicates a lower likelihood of off-target effects. My goal was to set the stage for engineering PSY to colonize tomato leaves and produce insecticidal dsRNA, thwarting any hornworms attempting to eat the plant.

Methods

Plant Colonization Assays

In order to assess how the Psy508 strain grew on tomato leaves over time, I collected leaf samples using a sterilized hole-puncher and tweezers. I grew an overnight liquid culture of Psy508 in Luria broth (LB) + 50 µg/mL kanamycin, concentrated this culture with a microcentrifuge, resuspended the resultant pellet in 10 mM MgSO4 to bring the optical density (OD) to 1.2, and then added 0.025% Silwet L-77 to act as a surfactant. I pipetted 30 µL of this solution onto the leaf samples and then transferred three leaf samples into filter bags each day, mixing them with 10 mM $MgSO_4$ to prevent osmotic lysis, and grinding them down with a pestle. The filter bags contained a fine mesh sheet between the two plastic sides of the bag, preventing any leaf debris from moving to the bottom of the bag while allowing liquids to pass through for collection. I then diluted the resultant liquid leaf sample several times in sterile saline, spot-plated each dilution onto plates of LB + 50 µg/mL kanamycin. I left them to incubate

at room temperature for two days. Afterwards, I counted the number of colonies in each dilution on the spot plates and used these counts to determine the actual colony forming units (CFU) per square centimeter on the plants themselves.

gfp Integration into PSY

To conjugate and integrate *gfp* into the genomes of Psy508 and Psy642, I inoculated liquid overnight cultures of the two PSY strains along with two Mu-free donor strains: one with the mini-Tn7-GFP plasmid and one with the pTNS2 transfer plasmid. After going through the appropriate washes, resuspending in saline, measuring their ODs, and diluting the more concentrated cultures to bring them all to the same OD, I mixed the strains and plated them on LB + 0.3 mM diaminopimelic acid plates. I used two of these plates: one for the Psy508 transformation and one for the Psy642 transformation. Each of these plates had one quadrant into which I pipetted 50 µL of the PSY strain. In the other three quadrants, I pipetted 50 µL of different combinations of the donors and recipients: 1:1:1 (16.67 µL of PSY), 1:2:2 (10 µL of PSY), or 2:1:1 (25 µL of PSY).

After allowing the plates to incubate at room temperature overnight, I scraped off portions of the three mixed-strain regions, washed them, resuspended them in 100 µL of saline, and then plated them on LB + 50 µg/mL kanamycin selective plates. These six plates incubated at room temperature until fluorescent colonies were observed. I picked fluorescent colonies to run 16S rRNA PCRs and sequenced them to confirm that their species was indeed *Pseudomonas*.

dsRNA-producing Plasmid Construction

The plasmids meant to produce *vATPaseE* double-stranded RNA comprised three main components: a small portion of the *vATPaseE* gene, two promoters, and a backbone incorporating spectinomycin (SPEC) antibiotic resistance.

The latter two components were already combined into one plasmid called pBTK800, gifted by Sean Leonard. The plasmid was modeled after similar broad-host range plasmids designed such that different fluorescence reporter genes and antibiotic resistance cassettes could be combined using Golden Gate assembly. These parts and plasmids were previously used to engineer bee gut symbionts (Leonard et al., 2018). In pBTK800, a *gfp* dropout sequence was located between the two inward-facing promoters. This combination of SPEC resistance and GFP fluorescence were used as markers to determine the presence or absence of the plasmid.

<u>*vATPaseE* Insert Creation</u>

We ordered a clutch of 30 hornworm eggs from Carolina Biological and reared them on artificial diet or tomato plant matter (grown in lab). When the larvae reached their fifth instar, one was randomly chosen to supply the genomic information necessary to create the *vATPaseE* insert. An mRNA extraction kit was used to obtain genomic mRNA from the selected hornworm, and then I used a reverse transcription kit to convert this into a cDNA genome.

Overlap extension primers were designed to amplify two small portions of *vATPaseE* to test the mortality rates produced by targeting two different regions of the same gene:

Table 1: Primers Isolating Portions of *vATPaseE*

Insert #	Forward Primer	Reverse Primer	PCR Annealing Temperature
1	GCATCGTCTCATCGGTCTCA**TATG**AAGGCCAAGATCAAGAGGA	ATGCCGTCTCAGGTCTCA**GGAT**CCGAACAGCGCGTTACGGA	62.1 ˚C
2	GCATCGTCTCATCGGTCTCA**TATG**AATAACCTACAGCATGGCGC	ATGCCGTCTCAGGTCTCA**GGAT**ATGAGAGTGACCAGGAGA	59.6 ˚C

The 5′ to 3′ sequence of the forward and reverse primers for inserts 1 and 2. The bolded portion of the primers represent BsaI binding sites included in the overlap extension primers enabling Golden Gate assembly. The far-right column shows the combined annealing temperature when using Phusion DNA Polymerase.

The resultant PCRs were run on 1% agarose gels, and their bands were purified using a gel extraction kit.

Golden Gate Assembly

With the insert sequences successfully made, we proceeded to insert them into pBTK800 using Golden Gate Assembly. The original primer designs included small, four bp sequences that work with the restriction enzyme BsaI to assemble the insert into the backbone plasmid (bolded in *Table 1*): TATG for the forward primers and GAGT for the reverse primers. 10 total fmol of DNA backbone and 20 fmol from an insert were required for each reaction, and thus the length and concentration of each DNA part were used to calculate the total volume to be added to the reaction. The two reactions were as follows:

Table 2: *vATPaseE* Insert 1 Plasmid Creation

Reagent Name	Volume (µl)
Backbone pBTK800 Plasmid	1.85
Insert 1 (231 bp)	0.24
T4 DNA Ligase Buffer	1
T4 DNA Ligase	1
BsaI	1
diH_2O	4.91
Total	10

Reagent volumes for the Golden Gate reaction to produce the insert 1 plasmid.

Table 3: *vATPaseE* Insert 2 Plasmid Creation

Reagent Name	Volume (µl)
Backbone pBTK800 Plasmid	1.85
Insert 2 (419 bp)	0.28
T4 DNA Ligase Buffer	1
T4 DNA Ligase	1
BsaI	1
diH_2O	4.87
Total	10

Reagent volumes for the Golden Gate reaction to produce the insert 2 plasmid.

Final Transformations

These two final assemblies were transformed into electrically competent Psy508 and Psy642 cells using a protocol adapted from Blanchard et al. 2017 (Blanchard et al., 2017). The electrocompetent cells were chilled on ice, shocked at 12.5 kC/cm, and recovered in shaking LB media at room temperature for one hour. I plated the transformed cells onto LB + 120 µg/mL SPEC plates. I picked non-fluorescent colonies and grew these in five mL LB + 600 µg SPEC liquid tubes. I then used Sanger sequencing to verify that the picked colonies contained the desired final plasmid, and 16S primers to confirm that the picked colonies were indeed PSY.

Whyard Mortality Study Replication

Artificial Diet ("Chow") and Dish Creation

When feeding the hornworms on artificial hornworm diet ("chow"), I placed them inside petri dishes lined with chow on the bottom, a damp paper towel on top, and air holes through the lid.

To create the plates, I melted four airholes of two mm diameter in the lids of standard petri dishes using a soldering iron. I then surrounded the rim of the lid with hot glue and placed white mesh over the lid to prevent hornworms from escaping.

To create the chow, I mixed 19.4 g of chow powder ordered from Wormyworms on Amazon with 80.6 mL of distilled water in a 125 mL bottle and then stirred the contents with a magnetic stir bar. I microwaved this bottle at medium power until the liquid became a bright yellow color, then immediately poured the chow out into the petri dishes, using just enough to cover the bottom of the plates.

I then dampened a paper towel with distilled water and pressed it between the lid and the plate. To maintain dampness, I sprayed the paper towel with distilled water every three to four days. Each plate could fit a dozen first instar larvae comfortably for one week.

in vitro Transcription

Whyard et al. 2009 laced hornworm chow with *in vitro* transcribed *vATPaseE* dsRNA (Whyard et al., 2009). I used three sets of primers (detailed in *Table 4*) to amplify enough DNA template to create dsRNA for the two inserts. The first set (abbreviated as "Long" in *Table 4*) amplified the *vATPaseE* gene from our *M. sexta* cDNA using Phusion DNA polymerase. The second and third sets amplified the insert 1 and insert 2 subsections of *vATPaseE* using OneTaq

DNA polymerase and added inward-facing T7 RNA polymerase promoters to each end of the PCR product. After amplifying the correct regions, I used one µg of each sequence with a HiScribe T7 RNA Synthesis Kit (New England Biolabs) in order to make the final dsRNA.

Table 4: *vATPaseE in vitro* dsRNA Creation

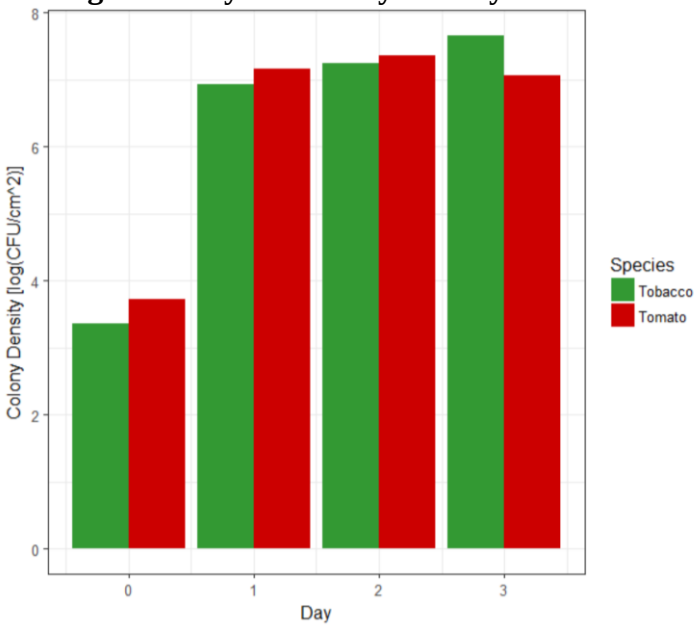
Insert #	Forward Primer	Reverse Primer	PCR Annealing Temperature (Polymerase)
Long	GTTTTTCGTGTTAGTGGTGC	AACGAGTGAATGTGGGTCAC	60.7 ˚C (Phusion)
1	ATGTAATACGACTCACTATAGGAAGGCCAAGATCAAGAAGGA	ATGTAATACGACTCACTATAGGCCGAACAGCGCGTTACGGA	48 ˚C (OneTaq)
2	ATGTAATACGACTCACTATAGGAATAACCTACAGCCATGGCGCTCAG	ATGTAATACGACTCACTATAGGATGAGAGTGACCAGGAGATCTGAGT	56 ˚C (OneTaq)

The 5′ to 3′ sequence of the forward and reverse primers for vATPaseE*, insert 1, and insert 2 in preparation for* in vitro *transcription. The far-right column shows the combined annealing temperature when conducting PCR, as well as the polymerase enzyme used.*

Results

PSY Grows and Sustains Colonies on Tomato Leaves

Figure 1: Psy508 Colony Density over Time

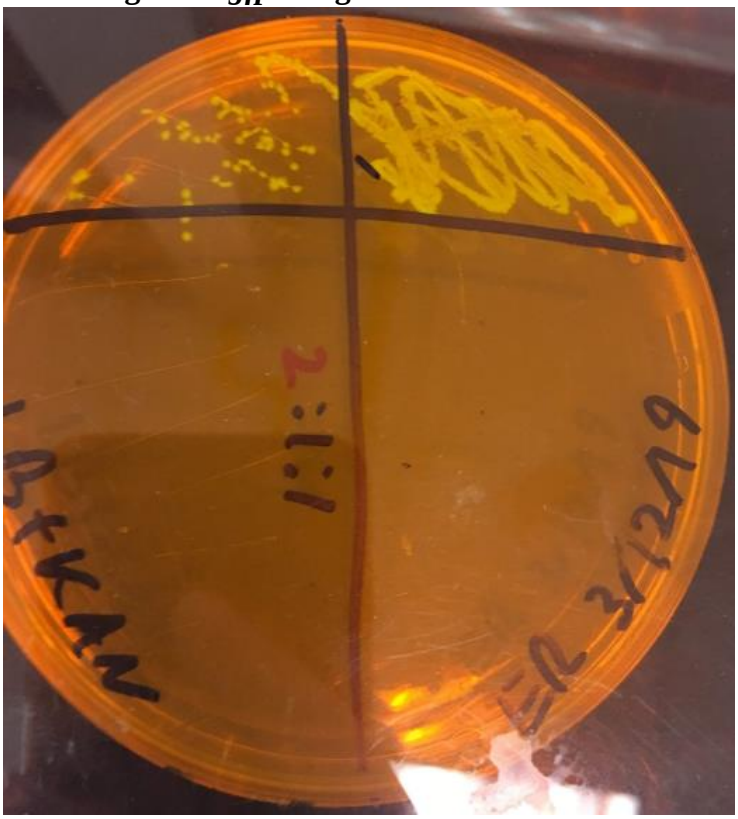


The vertical axis represents colony density on leaves, converted into CFU per square centimeter. The horizontal axis represents time in days. Psy508 density on tomato plants is represented in red, while density on tobacco plants is represented in green.

Plant symbionts could be superior dsRNA producers to *E. coli* because of their ability to non-pathogenically colonize crop plants for long periods of time. To confirm that the PSY strains can maintain colonies without inducing adverse effects on the host plant, I inoculated leaf discs with Psy508 strains and measured their cell density daily. *Figure 1* demonstrates that Psy508 was able to maintain steady colonies on tomato and tobacco leaves for at least three days. Psy508 on tomato grew to slightly higher densities on average but not by a statistically significant amount ($p = 0.53$). These measured densities were comparable to those found in past literature (Mohr et al., 2008).

Successful Integration of *gfp* into PSY Genomes

Figure 2: *gfp* Integration Confirmations



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Pseudomonas sp. BcB153 16S ribosomal RNA gene, partial sequence	143	143	40%	2e-30	94%	EU680978.3
☐	Pseudomonas fluorescens partial 16S rRNA gene, isolate BEG218	143	143	40%	2e-30	94%	LT628113.1
☐	Pseudomonas sp. BEG069 partial 16S rRNA gene, isolate BEG069	143	143	40%	2e-30	94%	LT628171.1
☐	Pseudomonas syringae pv. atrofaciens strain GN-In 16S ribosomal RNA gene, partial sequence	143	143	40%	2e-30	94%	MK141010.1

Top: Isolation plate for integrating gfp *into the Psy508 genome. In order to visualize GFP fluorescence, these images were taken under a blue light transilluminator—with an orange filter for eye protection.*
Bottom: The NCBI BLAST results matching Psy642 fluorescent colonies to particular organisms

In order to better track the presence of PSY bacteria, we wanted to have the strains express GFP. While there are many plasmids that already encode expression of GFP, we decided to integrate *gfp* into the genome of our PSY strains. Plasmids with traits such as GFP that do not confer fitness advantage are often lost—unless there are selective pressures, such as antibiotics, that require the strain to maintain the plasmid. Because we do not intend to spray crop plants with antibiotics, integration into the strains' genomes would be preferable.

I successfully integrated a gene expressing GFP into the genomes of both Psy508 and Psy642. Some select results from this process are shown in *Figure 2*. On top, the fluorescence of individual Psy508 colonies are visible after the GFP expression gene has been conjugated into

the bacteria. After picking such colonies, I sequenced the 16S region of the genome and entered the results into NCBI's BLAST database to check that the fluorescent colonies were not contamination from another species. The bottom of *Figure 2* shows a confirmation that the fluorescent colonies were indeed *Pseudomonas*, as indicated by an E value of 2×10^{-30}. The rRNA sequence for Psy508 and Psy642 are not available in this database, which is why the top result was not a perfect match, nor listed under these particular strain names.

Creation and Transformation of dsRNA-producing Plasmids

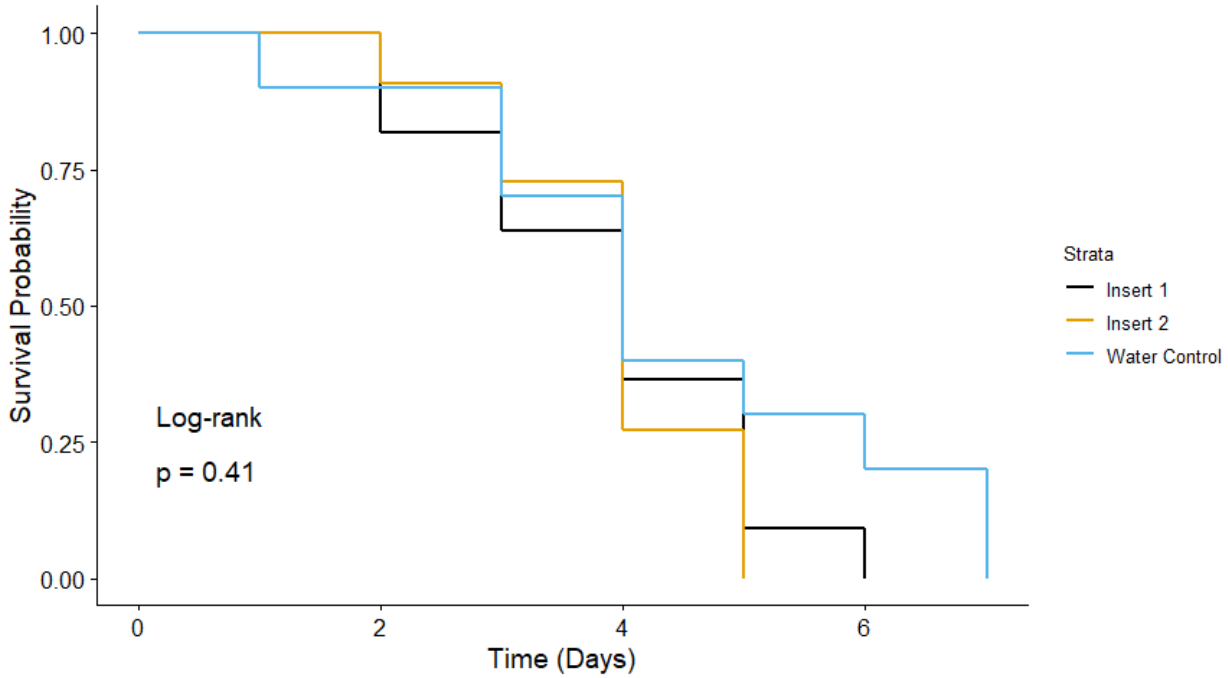
Figure 3: Integrated GFP Expression in PSY Strains

Left: gfp *integrated into Psy508 (top) and Psy642 (bottom). Psy508 contains the plasmid encoding* vATPaseE *insert 1 and SPEC resistance, and Psy642 might.*
Right: gfp *integrated into Psy508 (top) and Psy642 (bottom), both of which contain the plasmid encoding* vATPaseE *insert 2 and SPEC resistance.*
All plates are LB + SPEC agar, fluorescing under a blue light transilluminator with an orange cover.

I tried transforming both of the *vATPaseE*-dsRNA-containing plasmids into the Psy508 and Psy642 strains that had *gfp* integrated into the genome, creating a total of four strain combinations. The results of these attempted transformations are shown in *Figure 3*. All strains were capable of growing on SPEC-containing plates, and all strains fluoresced.

Whyard Mortality Study Replication

Figure 4: Tobacco Hornworm Survival over Time



I fed tobacco hornworms diet laced with either water (blue, N = 16), vATPaseE *insert 1 dsRNA (black, N = 17), or* vATPaseE *insert 2 dsRNA (yellow, N = 16) and then recorded their deaths over the course of seven days. The y-axis shows the survival probability, or percentage of hornworms still alive in each experimental group. The x-axis shows the number of days since the beginning of the experiment.*

To confirm *vATPaseE* dsRNA's lethal effect on tobacco hornworm larvae, I attempted to replicate a survival analysis conducted by another research group (Whyard et al., 2009). This study successfully increased the mortality rates of *M. sexta* caterpillars by lacing their food with *in vitro* transcribed dsRNA for *vATPaseE*. *Figure 4* above shows the survival curve of tobacco hornworms exposed to control water, insert 1 dsRNA, and insert 2 dsRNA. While hornworms in the experimental groups exhibited slightly reduced survival, the differences among all three groups were not significant (*Table 5*). It is possible that these nonsignificant results are partly due to the small sample size used in this replication, and that a larger sample size would reveal a subtle effect from the dsRNA. Additionally, I individually tested the significance of each insert's effects compared to controls. *Table 5* summarizes the outcome of each statistical test:

Table 5: P Values for Tobacco Hornworm Mortality Assay

Conditions	N	Statistical Analysis	P Value
All Three Conditions	49	Cox	0.3
All Three Conditions	49	Kaplan-Meyer	0.4
Water vs Insert 1	33	Cox	0.3
Water vs Insert 1	33	Kaplan-Meyer	0.3
Water vs Insert 2	32	Cox	0.2
Water vs Insert 2	32	Kaplan-Meyer	0.3

I used both Cox and Kaplan-Meyer tests to measure if the hornworm death rates significantly differed between caterpillars in each condition. Column 1 states which groups were compared. Column 2 states the number of total hornworms used in the analysis. Column 3 states which statistical analysis was used to compare group outcomes. Column 4 states the resultant p value of each analysis.

Discussion

The final goal of this study was to certify the potential of native crop symbionts to produce *vATPaseE* dsRNA from a plasmid to induce *M. sexta* mortality. I confirmed that one strain of *Pseudomonas* is capable of colonizing tomato and tobacco leaves for several days without any detectable deleterious effects. In other studies, Psy508 grew at comparable rates and to comparable densities (Mohr et al., 2008). This data indicates that these PSY strains have potential to colonize crop plants for extended periods of time in order to deliver their dsRNA payloads.

This is the first time that a gene encoding green fluorescence has been successfully integrated into the genome of Psy508 and Psy642. The distinct fluorescence on the selective plates, as well as the sequencing results indicating that these were indeed the correct strains and not contamination, confirms this result. This fluorescence will enable further studies to track and distinguish the engineered PSY strains from other microbes that may colonize leaves of experimental plants.

Theoretically, the bacteria growing on LB + SPEC plates should all be *Pseudomonas* and should all have the transformed plasmids: they are all fluorescent, indicating that these are the intended integrated GFP strains; and they are all resistant to the spectinomycin on the plates, implying presence of the antibiotic resistance gene on the plasmids. However, I cannot conclude that all of these transformations were successful, because the Psy642/insert 1 plasmid combination had inconclusive sequencing results. I confirmed that the other three combinations were successful via sequencing. These fluorescent strains will be easier to track and distinguish than wildtype PSY, and the integration of *gfp* into their genomes will be more stable than PSY containing GFP-producing plasmids. The dsRNA-producing plasmids in these three (potentially four) strains may be able to induce hornworm caterpillar mortality if ingested. Future studies could employ these strains to confirm this hypothesis. If confirmed, it would also be beneficial to integrate the dsRNA expression cassette into the genomes of these strains in order to maintain dsRNA expression for a longer period of time.

There was no significant difference in hornworm death rates between groups exposed to a water control versus those exposed to insert 1 or insert 2. There are several reasons that may explain why we failed to replicate the Whyard study's results, which had demonstrated significant hornworm mortality induced by dsRNA (Whyard et al., 2009). Firstly, there was significant mold contamination, which may have spoiled the hornworms' food supply or else harmed the hornworms themselves. Secondly, some of the hornworms did not appear to consume much chow, if at all, and so may have died of starvation or disease before getting the chance to ingest the dsRNA. Lastly, it was sometimes difficult to declare exactly when a hornworm died: sometimes they would be unresponsive and motionless one day, but fine the next; other times, the hornworms would be unresponsive for several days before seeming obviously dead (with a desiccated, discolored appearance).

After consulting another tobacco hornworm researcher, Dr. Toby Hammer, we determined that there are several things future researchers could do differently to improve this experiment. Some of the hornworms seemed to starve to death despite having plenty of food and would simply stop moving. Dr. Hammer suggested that the hornworms may have been dying of disease at an early instar, and that future researchers should soak hornworm eggs in 10% bleach before placing them in their containers. This would also help prevent mold contamination. Additionally, future researchers can prevent contamination and disease by providing the hornworms with fresh food every day, keeping as few hornworms in a container as possible (to prevent cannibalism), and cleaning frass out of their containers daily.

Conclusion

Conventional chemical insecticides frequently have negative off-target effects on other organisms. Researchers have proposed using RNAi to target essential genes that are specific to agricultural pests. This could mitigate harmful effects on other insects or humans. However, dsRNA is unstable outside of cellular environments, and would quickly deteriorate if simply sprayed over a crop field. Here, I present the characteristics of an ideal insecticide alternative. I confirm that a non-pathogenic strain of PSY colonizes tomato and tobacco leaves to high titers for at least 72 hours; integrate a GFP expression cassette into two PSY genomes for effective colonization tracking; and construct two plasmids that express portions of *vATPaseE* and transform them into PSY. These results suggest that using symbiotic leaf bacteria to continuously produce and stabilize insecticidal dsRNA may be feasible. The tools I have developed determine that trials on live pests and live tomato plants may be just around the corner. In the future, the stability, sustainability, and cost effectiveness of dsRNA insecticides could allow us to protect crop plants without sacrificing human or environmental health.

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